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CHANGES OF THE CHICKEN CHORIOALLANTOIC MEMBRANE AND THE BEHAVIOUR OF TRANSPLANTED GLIOBLASTOMA

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ABSTRACT

Glioblastoma is the most common brain malignancy and is marked by an extremely poor prognosis, despite advances in surgical and clinical neuro-oncology. Glioblastomas are very heterogenic in their biological and morphological features and they are widely investigated.

Existing in vivo glioblastoma models are based on the inoculation of glioma cells or cell lines into the rodent brain, the dog brain or the use of transgenic mice causing spontaneous tumors. These models suffer from the variable growth rate and poor penetration, and are limited by the difficulty of obtaining morphological data. In our research we suggested the model in which the native human glioblastoma was transplanted into the chicken embryo chorioallantoic membrane. The glioblastoma was transplanted into the embryo's chorioallantoic membrane on the seventh – ninth day, when it was fully developed and could ensure the nutrition of the tumor. Transplantation was successful if the glioblastoma survived at least for 24 hours together with the embryo. The chorioallantoic membrane after transplantation showed thickenning. Between 48 and 120 hours after transplantation the thickness of the membrane changed from 2x to 5x. Starting from 144 hours after transplantation the thickness of the membrane diminished. The tumor transplanted into the chorioallantoic membrane ingrows in it in the zones where the epithelium of the membrane was mechanically removed. The tumor keeps its proliferative activity until 48 hours of transplantation,

afterwards the proliferative activity is noticed in the chorioallantoic membrane until 120 hours of transplantation. This shows that the main processes take place in the zone where the tumor adheres to the chorioallantoic membrane.

The human glioblastoma transplanted on chicken chorioallantoic membrane repeated all the essential stages of tumor growth, which are also typical of other mammal models. This model reflects the morphological and biological features of the glioblastoma, allows to evaluate the invasivity, the progress of the tumor, and to investigate new medicines.

Key words: *chorioallantoic membrane, glioblastoma, chicken embryo.*

INTRODUCTION

Glioblastoma is the most common brain malignancy and is marked by an extremely poor prognosis, despite advances in surgical and clinical neuro-oncology [1, 12, 19, 20]. Glioblastomas are very heterogenic in their biological and morphological features. Despite the major advances in science, a lot of questions regarding glioblastomas are still without an answer – what are the main etiological and risk factors of glioblastoma, what molecular mechanisms are involved, what factors are responsible for poor prognosis and resistance to therapy [7]. The factors mentioned above constitute factors the reason why these malignancies are still so much investigated.

Glioblastoma models existing in vivo are based on the inoculation of glioma cells or cell lines into the rodent brain, the dog brain or the use of transgenic mice causing spontaneous tumors. However, these models suffer from the variable growth rate and poor penetration, and are limited by the difficulty of obtaining morphological data. These models do not reflect properly the interaction between the tumor and the host that occurs in the human, the accurate invasion processes, the vasculature, the gene expression profiling, and stroma interactions [2, 4, 6, 9].

The chicken embryo chorioallantoic membrane assay is a well established method for studying cancer, cell biology, immunology and genetics. Heterologous tissue transplantation into the chorioallantoic membrane of the fertilized chicken egg was first reported by Murphy in 1912. In 1934 Burnett and Ferry showed that many viruses can be grown on the chicken chorioallantoic membrane [15]. This extra-

embryonic well vascularised membrane is created by the fusion of chorion and allantois. The model of the chorioallantoic membrane is used for the investigation of growing tumors (lung, prostate, skin, brain) and viruses, the endometriosis research, the research of angiogenesis, for new medicines and therapeutical targets [3, 5, 8, 11, 13, 14, 17, 18, 21].

Nowadays the experimental models for tumors, especially for the glioblastoma, are under research. These new models should reflect the morphological and biological features of the glioblastoma, should allow to evaluate the invasivity, the progress of the tumor and to investigate new medicines. All these models should not be expensive. The model of the chicken chorioallantoic membrane has all the mentioned features. It is important that chicken embryos are immunodeficitic and there are no reactions of transplant rejection. This model for the glioblastoma research is used rarely [9, 16].

The objectives of this work: To evaluate changes of the chicken embryo chorioallantoic membrane, which appear after the transplantation of the glioblastoma and the changes of the membrane ensuring survival of the transplanted glioblastoma and to determine the invasivity of the glioblastoma into chicken chorioallantoic membrane.

MATERIALS AND METHODS

The glioblastoma tissue was taken from the patients (n=15) operated in the Department of Neurosurgery of *Lithuanian University of Health Sciences Kaunas Clinics.* All the patients had clinical and radiological diagnosis of the glioblastoma and gave the permission to participate.

The model of the chicken chorioallantoic membrane

Overall 300 of fertilized eggs (Hisex Brown, the Vievis Poultry Farm) were used for these particular experiments, 20 eggs were used for one tumor transplant. Overall fifteen tumors were transplanted into the chorioallantoic membrane; ten of them were glioblastomas (the tumor stands at the 4^{th} stage of malignancy according to the World Health Organization (WHO) classification), 200 eggs were used for the transplantation of glioblastomas into the chorioallantoic membrane. The diagnosis of the glioblastoma was confirmed by the pathologist.

Eggs were inserted into the incubator (SIEPMANN AB) for three days. The temperature in the incubator was constantly kept at 37.8 °C

and the humidity was 60%. The incubator was ventilated with the room air, while the eggs were turned back and forward every hour. The first incubation day was called the zero day. The eggs were taken out on the third-fourth incubation day and the rectangular 1×1.5 cm hole was screwed in their shell. The third-fourth day after incubation is the best time to make a hole, as the embryo has already formed, the chorioallantoic membrane begins to form, the protein is transparent and liquid, which makes it easy to take out with the disposable syringe. The hole was covered with a sterile plastic wrap in order to maintain humidity and so that the development and the vitality of the embryo could be observed. About 2 milliliters of protein was taken out from the blunt end of the egg with the sterile needle, so that the yolk did not reach the shell of the egg and the embryo was not hurt . The eggs were put back to the incubator until the seventh-ninth development day, when the chorioallantoic membrane forms, but they were not being turned anymore.

The glioblastoma tissue within 30 min was transported to the laboratory, cut into small pieces of 0.2×0.2 cm and transplanted into the chicken chorioallantoic membrane on the seventh – ninth day of the incubation. The chorioallantoic membrane in that period was fully developed and could ensure the nutrition of the tumor. The epithelium layer of the chorioallantoic membrane was destroyed with the sterile stick to improve the penetration of tumor cells [16]. After the transplantation, the hole in the shell was covered with the wrap again and the eggs with transplants were put back into the incubator. The tumors with the transplanted glioblastoma were observed every day and the vitality of the embryos and transplants was tested. The dead embryos with the transplanted glioblastoma were removed from the incubator immediately.

Histology and immunohistochemistry

Transplanted on chorioallantoic membrane tumors were cut together with the membrane during certain time intervals – every 24 hours. Tissue samples were fixed in 4% neutral buffered formaldehyde. The tissue was dehydrated, embedded into paraffin and 3–5 μm thick sections were performed. After that sections were stained with hematoxylin and eosin, and the imunohistochemistry for glial fibrillar protein was performed. Immunochemical staining allows to differentiate

the human tumor tissue from the chorioallantoic membrane tissue. Histological slides were observed with the optic microscope "Zeiss – standart 25" using the MC DX photocamera.

RESULTS

The macroscopic view of transplanted glioblastomas

Transplantation was successful if the glioblastoma survived at least for 24 hours together with the embryo. 158 (79%) transplants survived for 24 hours during the experiment, 130 (65%) of transplants survived for 48 hours, 105 (52.5%) transplants survived for 72 hours, 74 (37%) transplants survived for 96 hours, 54 (27%) transplants survived for 120 hours, 26 (13%) transplants survived for 144 hours, 18 (9%) transplants survived for 168 hours, 9 (4.5%) transplants survived for 188 hours (Table 1). It also should be noted that a part of embryos together with the transplant did not survive also because they were taken away for histological and immunohistochemical tests.

Changes of the chorioallantoic membrane

The chorioallantoic membrane is a very vascular fetal membrane composed of the fused chorion and the adjacent wall of the allantois. Different sizes of blood vessels are abundant in the mesenchyme and the chorioallantoic membrane is a good nutritional environment for the glioblastoma. (Figure1)

There are changes in the normal chorioallantoic membrane that take place during transplantation, but on the whole the membrane does not change obviously. Comparing the normal membrane after 48 hours and 120 hours after transplantation, it is seen that the width of the membrane remains almost the same. Small vessels remain under the epithelial layer, while large ones are concentrated in the mesenchymal layer. This position of vessels helps for tumor nutrition. Though the drying of the membrane starts after 120 hours of transplantation, microscopically only the mesenchymal layer seems denser. After 144 hours from the transplantation the membrane is thinner because of drying, while the

Figure 1. Normal chorioallantoic membrane. V – blood vessel, E – epithelium layer, M – mesenchymal layer, ChE – epithelium of the chorion. Magnification 40x. Scale bar – 20 μ.

epithelium has proliferated from both sides. After 188 hours of transplantation the membrane is thin, the epithelium layer has dried.
During incubation the chorioallantoic membrane becomes thicker not only in the glioblastoma fixation region, but also in the adjacent region of about 1 cm diameter. After 48 hours of transplantation the membrane becomes thicker 2x, after 72 hours the chorioallantoic membrane becomes thicker 4x, and after 120 hours – 5x (Figure 2). The membrane becomes more vascularised and the proliferation of the epithelium is noticed. The changes of the thickness of chorioallantoic membrane are shown in the Figure 3.

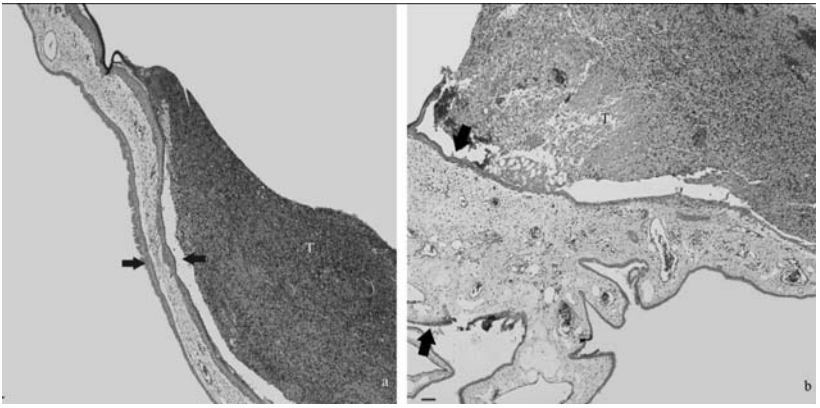

Figure 2. Thickness (arrows) of the chorioallantoic membrane after 48 hours (a) and 120 hours (b) from transplantation. T – tumor. Magnification 4x. Scale bar – 20 μ.

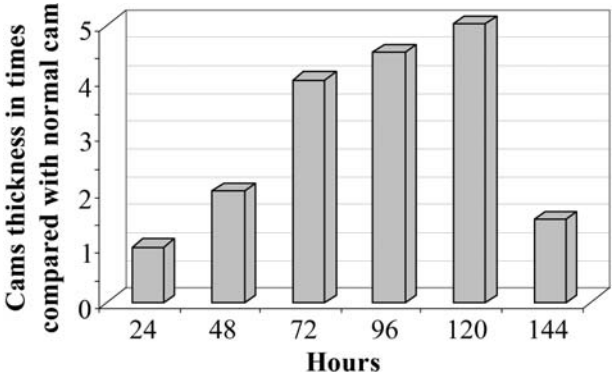

Figure 3. Thickness changes of the chorioallantoic membrane during different transplantation periods.

In the regions, where the epithelium of the membrane was removed, the reaction of the membrane is much more obvious – the small vessels are targeted to the epithelium and the transplanted tumor survives better, because the nutrition is better and the tumor cells pass the membrane easier. The small membrane vessels are entering the tumor, thus keeping it alive. We can distinguish the vessels of chicken from the nucleated erythrocytes.

In the regions, where the epithelium was not removed, the reaction of membrane is not so active, the vascularisation is smaller, epithelium proliferation is not so active.

Behaviour of the transplanted tumor

The tumor transplanted into the chorioallantoic membrane ingrows in it in the zones where epithelium was mechanically removed. The tumors keeps its proliferative activity until 48 hours of transplantation, afterwards the proliferative activity is noticed in the membrane until 120 hours of transplantation. This shows that the main processes take place in the zone where the tumor adheres to the chorioallantoic membrane.

After 48 hours of transplantation the week invasion of glioblastoma cells could be noticed, but the more noticeable invasion starts at 72 hours after transplantation (Figure 4). The borders of the tumor and the

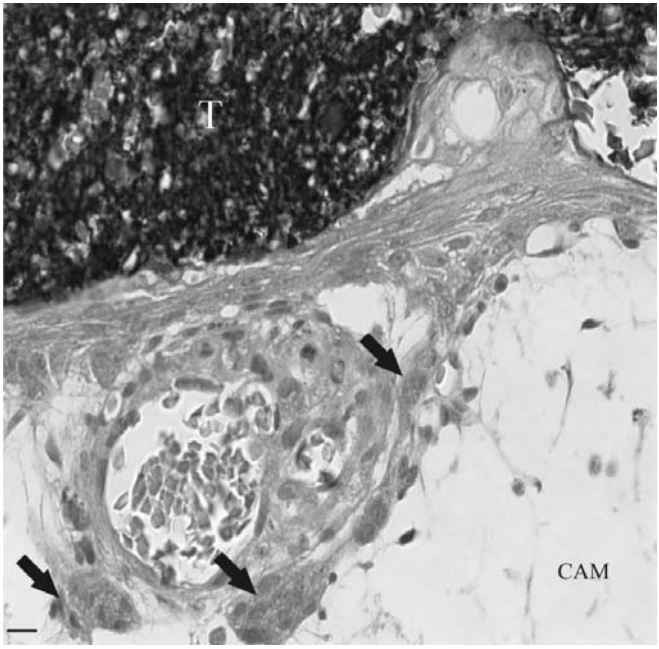

Figure 4. Invasion (arrows) of the transplanted glioblastoma (T) into the chorioallantoic membrane (CAM) after 72 hours of transplantation. Magnification 40x. Scale bar – 20 μ.

chorioallantoic membrane are still noticeable. The proliferation of epithelium is seen in the invasion zone. The fact that the cells invading the chorioallantoic membrane are of glial origin shows the positive immunohistochemical reaction to the glial fibriliar acid protein.

The most active invasivity of glioblastoma cells is seen at 120 hours after transplantation. Invading glioblastoma destroys the epithelium layer of the membrane and no more the boarders of the tumor and the membrane are distinguished (Figure 5).

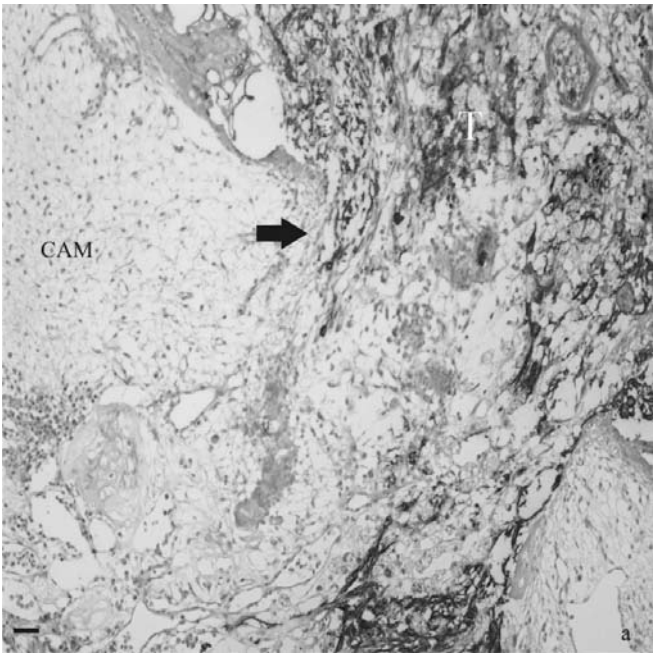

Figure 5. Invasion (arrows) of the transplanted glioblastoma (T) into the chorioallantoic membrane (CAM) after 120 hours of transplantation. Magnification 40x. Scale bar – 20 μ.

The glioblastoma invades the chorioallantoic membrane along its vessels. The glioblastoma vessels are also participating in the process by helping glioblastoma invasion. The formation of new glioblastoma vessels starts at 72 hours after transplantation, while being the most active at 120 hours after transplantation.

DISCUSSION

Referring to the data of our experiments, the tumors on the chorioallantoic membrane survived six days. Further survival of transplanted glioblastomas is limited because of the nourishing membrane becoming dry. This process takes place because of the natural process of embryo development – during the development of the embryo the outer layer of the chorioallantoic membrane is becoming dry and is not able to ensure sufficient nourishment of the transplanted tumor. The data obtained does not contradict other authors' data. Shoin, Yamashita et al. [16], who transplanted gliomas, also emphasize that the average survival of the tumor on the chorioallantoic membrane is seven days. The authors indicate that, wishing to prolong the time of tumor survival, it is possible to transfer it on the other chorioallantoic membrane. Other authors [9] do not indicate the duration of the tumor survival at all. Assessing the chorioallantoic membrane as an experimental model for the research of various anticancer medications, Vargas, Zeisser-Labouebe et al.[18] indicate that the experimental model has the same characteristics as the other models of mammals and tumors retain their growing characteristics; however, due to the development of the chicken embryo, the model is suitable only for the research of short duration. Therefore, most of the authors emphasize that the chorioallantoic membrane is a suitable model for short-term experiments, which coincides with our results obtained.

The growth of the tumor on the chorioallantoic membrane is accompanied by membrane thickening: the chorioallantoic membrane around the tumor changes significantly. The chorioallantoic membrane is significantly thicker in the area of tumor growth as well. The membrane starts thickening after 48 hours of transplantation, being the thickest at 120 hours from transplantation. The membrane became thicker 5 times compared to the normal chorioallantoic membrane. This coincides with other authors [9] who confirm that in the places where the tumor adheres to the chorioallantoic membrane, it gets thicker six times in comparison with the control group. These authors name this thickening as the edema of the membrane. In our case, this thickening was more proliferation than edema, because the number of cells and vessels in the membrane increased. The chorioallantoic membrane reacted to the transplanted tumor also with vascularisation and epithelium proliferation.

Glioblastoma cells invade into the chorioallantoic membrane, the process of invasion starts at 48 hours of transplantation and is most active at 120 hours of transplantation. Glioblastoma cells invaded the chorioallantoic membrane along membrane vessels. The membrane vessels grew into the tumor keeping it alive. According to Hagedorn, Javerzat et al. [9], tumor cells penetrated actively into the chorioallantoic membrane along the new blood vessels of chorioallantoic membrane which grew in the tumor. The transplants of melanoma also metastasized and melanoma cells penetrated into the chicken chorioallantoic membrane [10].

Glioblastomas transplanted on the chicken chorioallantoic membrane repeated all the essential stages of tumor growth, which are also typical of other glioblastoma mammal models.

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